Identification of the Pigment Responsible for the Blue Fluorescence Band in the Laser Induced Fluorescence (LIF) Spectra of Green Plants, and the Potential Use of This Band in Remotely Estimating Rates of Photosynthesis

Emmett W. Chappelle,* James E. McMurtrey III,† and Moon S. Kim*

*Laboratory for Terrestrial Physics, NASA Goddard Space Flight Center, Greenbelt
†Remote Sensing Research Laboratory ARS / USDA, Beltsville

The laser-induced fluorescence (LIF) of vegetation is being investigated in this laboratory for use as a technique for the remote detection of the effects of environmental stress upon vegetation, as well as for plant identification. The fluorescence band with a maximum at 440 nm, in conjunction with the chlorophyll bands with maxima at 685 and 740 nm, has been found to be a critical band in the development of algorithms for detecting stress, and identifying plant types. The identification of the plant constituent responsible for this band is vital to understanding the mechanism underlying its fluorescence changes in response to environmental and physiological changes. The identification was achieved as follows: The laser induced fluorescence (LIF) spectra of pure plant pigments were determined. Fluorescence bands with maxima at 420 nm, 440 nm, 490 nm, and 525 nm were observed for vitamin K_1, reduced nicotinamide adenine dinucleotide (NADPH), beta-carotene, and riboflavin, respectively. The LIF spectra of water extracts and acetone extracts of clover leaves were also measured. It was found that the blue fluorescence band was associated with the water extract. NADPH which is a water-soluble compound, and the water extract of clover had no fluorescence after oxidation by potassium ferricyanide, while the fluorescence of water insoluble vitamin K_1 was unchanged by the oxidizing agent. It was also found that the absorption maximum of NADPH was the same as the absorption maximum of the aqueous extract of clover. The above findings indicated that the compound responsible for the blue fluorescence at 440 nm is in the reduced state and is water-soluble. It was concluded that NADPH was responsible for the blue fluorescence at 440 nm. The strong linear relationship between the fluorescence at 440 nm and the rate of photosynthesis suggests the possible use of LIF measurements in the remote estimation of photosynthetic rates.

INTRODUCTION

The characteristics of the fluorescence spectra of green plants when irradiated with a pulsed nitrogen laser with an emission at 337 nm were found by Chappelle et al. (1984a; b; 1985; Chappelle and Williams, 1987) to be related to plant type, as well as with changes in the physiology of the plant resulting from environmental stress factors. The plant types whose LIF spectra have been determined by Chappelle et al. (1985) include herbaceous dicots, monocots, hardwoods, and conifers. The dicots and monocots have distinct fluorescence bands at 440 nm, 685 nm, and 740 nm, and

a minor band at 525 nm. The monocots differ from the dicots by virtue of having a much higher 440 nm to 685 nm ratio. Hardwoods and conifers have the same bands, but also have a strong band at 525 nm. Conifers, however, have no fluorescence band at 685 nm.

It was initially thought by these authors that the blue fluorescence band at 440 nm was due to reflected secondary emissions from the nitrogen laser. This possibility was ruled out by the use of a 380 nm cutoff filter at the entrance of the monochromator, and also by determining that the emission band at 440 nm was isotropic.

Algorithms utilizing the fluorescence bands at 440 nm and 525 nm, along with the chlorophyll a bands at 685 nm and 740 nm have been successfully used in the detection of certain types of environmental stress by Chappelle and Williams (1987) as well as in the identification of plant types (Chappelle et al., 1985). It is believed that an improved understanding of the mechanisms underlying changes in fluorescence as the result of stress, and physiological differences among plant types could be obtained if the compound responsible for the fluorescence at 440 nm could be identified. The identification of this compound might also allow the development of algorithms for the detection of additional vegetational changes.

A number of investigators have observed a blue fluorescence emission with a maximum near 440 nm when microorganisms such as yeast, algae, and photosynthetic bacteria were irradiated with ultraviolet light (Duysens and Amesz, 1957; Duysens and Sweep, 1957; Olsen et al., 1959; Olsen and Amesz, 1960). It was suggested by the above investigators that the blue fluorescence of microorganisms was due to bound reduced nicotinamide adenine dinucleotide (NADPH). No attempts at a positive identification were made. It was recently suggested by Goulas et al. (1990) that the blue fluorescence band seen in the higher plants upon ultraviolet irradiation might also be attributed to NADPH.

There are also other compounds present in plant matter which emit a blue fluorescence when excited by ultraviolet light. These include lignins and other phenolics as described by Fry (1979; 1982) and Lundquist et al. (1978).

Lignins and phenolics are predominately structural compounds and relatively inert chemically. Our investigations of the changes in the magnitude of the blue fluorescence band in re-

sponse to certain environmental stress conditions (Chappelle and Williams, 1987) indicated that the blue fluorescence emission was due to a chemically dynamic molecule which was likely involved in photosynthesis. The studies described here indicate that this is the case.

EXPERIMENTAL METHODS

These studies were conducted using greenhouse grown clover (*Trifolium pratense* L) and soybeans (*Glycine max* L). The clover was grown in soil with a full complement of nutrients necessary for optimal growth. The soybeans were grown in perlite with all of the necessary nutrients except that the nitrogen concentration was varied ($0.002M$–$0.0005M$ urea) to obtain plants with different chlorophyll levels, and thus different rates of photosynthesis. Both of these plant species were used 6 weeks after germination for the following measurements: 1) rates of photosynthesis, 2) LIF spectra of pure plant pigments, the intact plants, and extracts of the plants, where the plant constituents were extracted with either water or acetone, and 3) absorption spectra of the extracts.

Rate of Photosynthesis

The rates of photosynthesis of soybeans were determined with a LICOR Model 6200 infrared gas analyzer in the closed mode. The measurements were made in the laboratory with the light source consisting of a combination of water cooled low pressure sodium lamps and alkaline metal halide lamps operating at an intensity of approximately 1800 $\mu M/m^{-2} s^{-1}$. The ambient CO_2 concentration was 330 ppm. The temperature within the photosynthesis chamber did not exceed 27°C, while the relative humidity was 58%.

LIF Spectra

A Molectron UV22 pulsed nitrogen laser was employed as the fluorescence excitation source. This laser, which emits at 337 nm, was operated at 30 Hz with a power output of 9 mJ per pulse (pulse width = 10 ns). The distance from the laser beam exit to the sample was 1 m. The sample area irradiated was defined by a 2.5 cm^2 aperture drilled in a copper nonfluorescent plate. The intact leaf

was taped to the back of aperture while the extracts and pure pigment solutions were in quartz cuvettes attached to the aperture. The fluorescence emission was collected and collimated by an f2.8, 180 mm telescope and focused on the entrance slit of a monochromator which scanned the fluorescence emission from 400 nm to 800 nm. The scan duration was 50 s. Any reflected laser beam emission at 337 nm was prevented from entering the monochromator by placing a 380 nm cutoff filter at the entrance of the monochromator. The fluorescence intensity as a function of wavelength was detected with a red sensitive GaAs(gallium arsenide) photomultiplier tube. A gated boxcar averager, triggered by an internal pulse generator, was used to capture the pulsed D.C. signal from the photomultiplier. The signal was fed, via an A/D converter into a computer. Data acquisition software was used for generating a real time spectra, and also data storage. The spectral intensities are expressed in relative fluorescence intensity units (RFI) (Chappelle et al., 1984).

Absorption Spectra

A Perkin-Elmer dual beam computer assisted spectrophotometer with a resolution of 1 nm was used for determining the absorption spectra of the pure pigments and extracts. The samples were scanned from 300 nm to 750 nm.

Preparation of Extracts and Pure Pigments

Those pigments soluble in organic solvents were extracted from clover leaves by extracting 5 g of leaf material in 100 mL of acetone for 12 h in the

dark at room temperature. The water-soluble components of clover were isolated by homogenization of 5 g of leaf material in 100 mL of water followed by centrifugation at 50,000 RPM and decanting the supernatant solution containing the pigments. Pure chlorophyll a, beta-carotene, and vitamin K_1 were dissolved in acetone $(1 \times 10^{-4} M/L)$. The water-soluble pure pigments, reduced nicotinamide adenine dinucleotide (NADPH), and riboflavin, were dissolved in water at the same concentration as above.

RESULTS AND DISCUSSION

Absorption Measurements

The absorption spectra of chlorophyll a, nicotinamide adenine dionucleotide (NADPH), vitamin K_1, riboflavin, and beta-carotene are shown in Figure 1. These pigments are all present in plants at significant concentrations. All of the pigments absorbed at 337 nm; the emission wavelength of the nitrogen laser. Thus, they are all capable of fluorescence upon irradiation by the laser.

LIF Measurements

The fluorescence spectra of an intact clover leaf is shown in Figure 2. Fluorescent bands were observed at 440 nm, 680 nm, and 740 nm. A small band was also seen at 525 nm. It was established by Kok (1976) that the fluorescence bands at 680

Figure 1. Fluorescence spectra of intact clover leaves obtained when irradiated with a pulsed nitrogen laser at 337 nm. The spectra were taken on the leaves of plants while still in their pots.
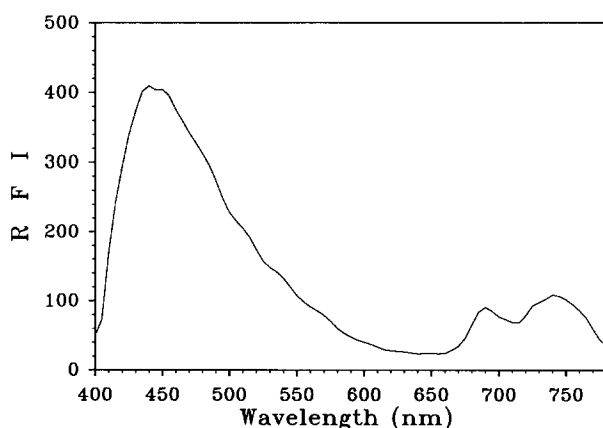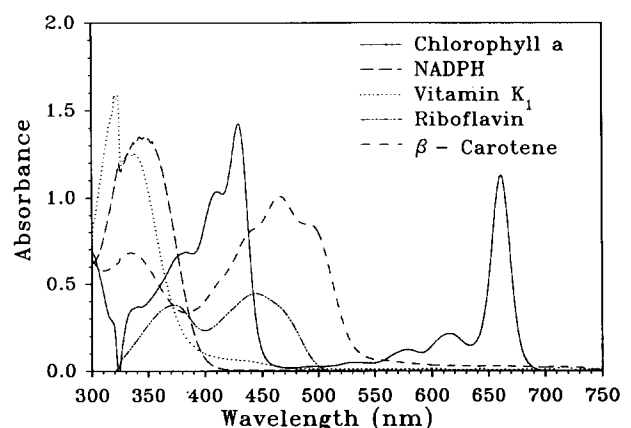
Figure 2. Absorption spectra of pure plant pigment solutions. The concentration of each was $1 \times 10^{-4} M$. Chlorophyll a, vitamin K_1, and beta-carotene were dissolved in acetone, and NADPH and riboflavin were dissolved in water. The spectra were obtained using a high resolution Perkin–Elmer double beam spectrophotometer.
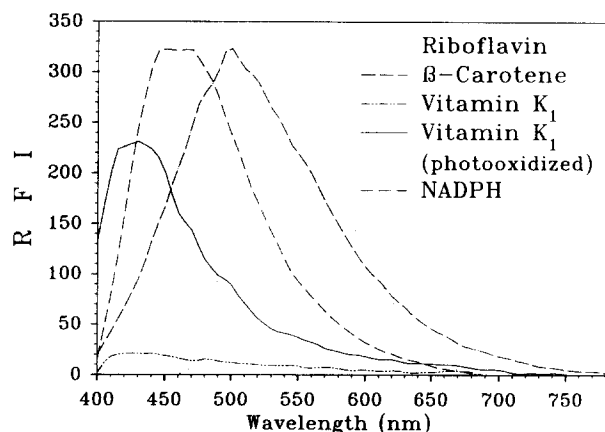
Figure 3. Fluorescence spectra of pure plant pigments obtained when irradiated with a pulsed nitrogen laser. The pigment concentrations were adjusted to give comparable fluorescence values.

Figure 4. Fluorescence spectra of aqueous extract and an acetone extract of clover obtained when the extracts contained in quartz cuvettes were irradiated with a pulsed nitrogen laser. Each extract was prepared from 5 g of leaf material in 100 mL of extracting agent.

nm and 740 nm were due to species of chlorophyll a. The fluorescence spectra of riboflavin, beta-carotene, vitamin K_1, oxidized vitamin K_1, and NADPH are given in Figure 3. It should be noted that vitamin K_1 fluoresced only after photooxidation by the laser beam. It was seen that the compound with a fluorescence maxima closest to 440 nm was NADPH. The fluorescence maxima of vitamin K_1, beta-carotene, and riboflavin were found to be at approximately 420 nm 490 nm, and 525 nm, respectively. It thus, appeared that the two most likely candidates responsible for the blue fluorescence at 440 nm were NADPH and vitamin K_1. Riboflavin, which is present in plants primarily in the forms of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), could be responsible for the small fluorescence band seen at 525 nm. This band has been shown by Chappelle et al. (1985) to be much more pronounced in conifers and hardwoods.

A comparison of the fluorescence spectra of an aqueous extract of clover leaves and that of an acetone extract is shown in Figure 4. It was observed that virtually all of the blue fluorescence was in the aqueous extract along with a trace of chlorophyll fluorescence. Almost all of the fluorescence seen in the acetone extract was due to chlorophyll a at 670 nm, with only a trace of the blue fluorescence band. It can be concluded that the compounds responsible for the blue fluorescence are water-soluble.

The solubility of the material associated with the blue fluorescence indicated the possible implication of NADPH and riboflavin in the blue fluorescence emission. It is believed that riboflavin is
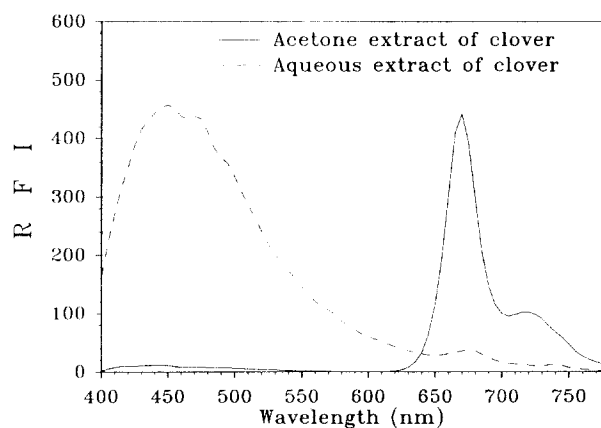
the compound responsible for the fluorescence at 525 nm, but its fluorescence maximum is so far removed from 440 nm that it is unlikely that it makes a significant contribution to the band at 440 nm. It is possible, however, that vitamin K_1 could be present in plant tissues as a water soluble complex.

Effect of Oxidation State on LIF

Nicotinamide adenine dinucleotide phosphate (NADP) fluoresces only in its reduced state (NADPH). This is shown in Figure 5, where after chemical oxidation by potassium ferricyanide, there was approximately 70% reduction in the fluorescence of NADPH at 440 nm. It was found that the 440 nm fluorescence band of the aqueous extract of clover behaved in a similar fashion after oxida-

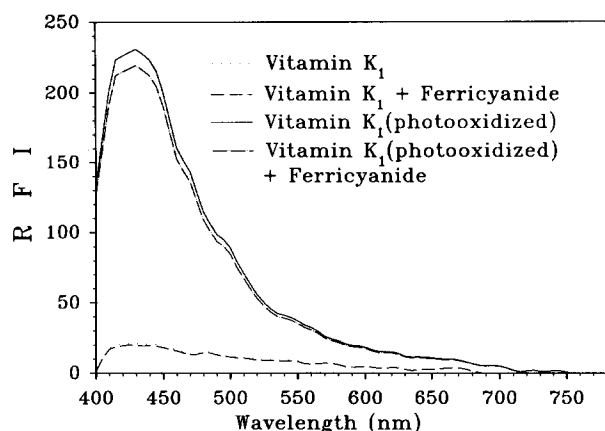*Figure 5.* Effect of oxidation by potassium ferricyanide on the LIF of NADPH and the aqueous extract of clover.
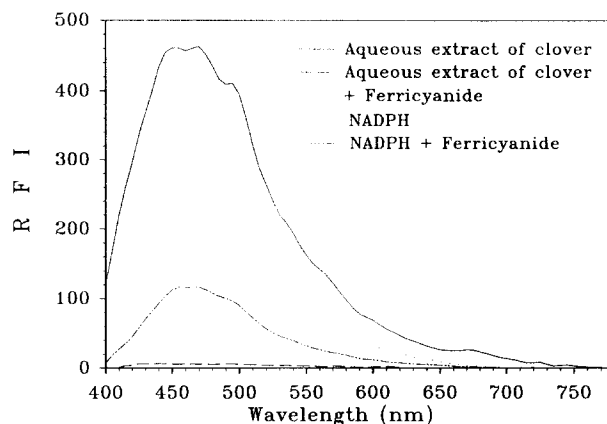
Figure 6. Effect of oxidation by potassium ferricyanide on the LIF of vitamin K_1 and photooxidized vitamin K_1. Vitamin K_1 was photooxidized by irradiation with the nitrogen laser for 5 min.

tion with potassium ferricyanide. It was shown earlier (Fig. 3) that vitamin K_1 was capable of fluorescence only after photooxidation by irradiation from the nitrogen laser for 5 min. It was found as shown in Figure 6 that there was no increase or decrease in the fluorescence of non-photooxidized vitamin K_1 in the presence of potassium ferricyanide. There was only a slight change in the fluorescence of photooxidized vitamin K_1 after exposure to potassium ferricyanide. It was further shown (Fig. 7) that exposure of the aqueous extract of clover and the NADPH solution to the photooxidizing effects of the laser beam for 5 min caused no changes in the level of their fluorescence. These experiments established that the oxidation-reduction properties of vitamin K_1 were different from those of NADPH and the aqueous extract, with vitamin K_1 fluorescing only in the

Figure 7. Effect of nitrogen laser irradiation on the LIF of NADPH and the aqueous extract of clover. NADPH and the aqueous extract were exposed to the laser beam for 5 min.
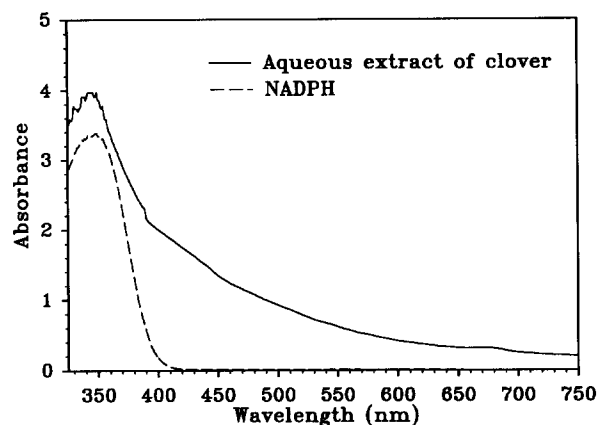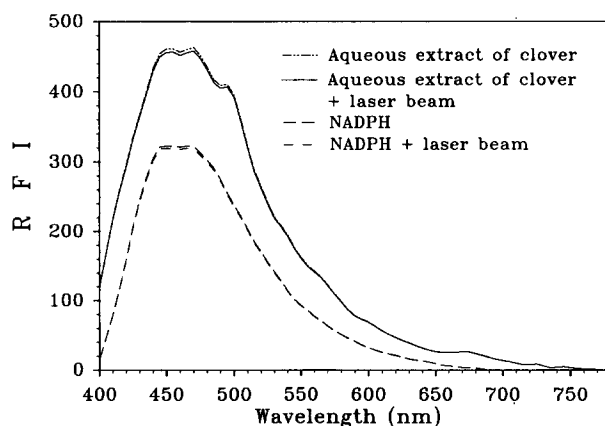

Figure 8. A comparison of the absorption spectra of NADPH and the aqueous extract of clover. The concentration of NADPH in water was $1 \times 10^{-3}M$.

oxidized state, whereas NADPH and the aqueous extract of clover exhibited fluorescence only in the reduced state.

Comparison of Absorption Maximum of Aqueous Extract and NADPH

The absorption maximum of NADPH was compared with the absorption maximum of the aqueous extract of clover leaves (Fig. 8). The absorption maximum were identical (440 nm). At this point it was concluded that the water-soluble compound responsible for fluorescence at 440 nm was NADPH.

The establishing of NADPH as the soluble compound responsible for fluorescence at 440 nm does not, however, rule out the possibility that long chain insoluble polymeric compounds such as lignins make a significant contribution. This possibility will require further study.

Significance of NADPH as Blue Fluorescent Pigment

NADPH plays a vital role in photosynthesis as it the primary energy sink in photosystem I whereby NADP is reduced to NADPH by accepting electrons from chlorophyll P700 (Okamura et al., 1982). It subsequently functions as an electron donor during the dark reactions of photosynthesis where CO_2 is reduced to sugar. The steady state concentration of NADPH should be a positive function of the rate of photosynthesis. This was validated by a regression of the fluorescence of soybeans at 440 nm against the rate of photosynthesis (Fig. 9). The
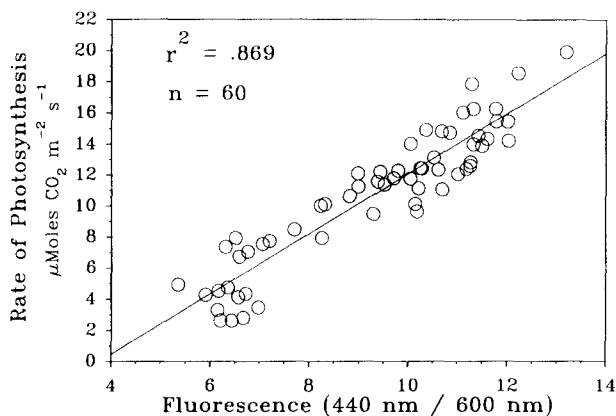
Figure 9. The rate of photosynthesis of soybeans versus LIF at 440 nm/600 nm.

fluorescence at 440 nm was normalized by the fluorescence at 600 nm (minimal fluorescence of the LIF spectra). A strong linear relationship between the rate of photosynthesis and the fluorescence at 440 nm is shown ($r^2 = 0.869$). This linear relationship of the blue fluorescence at 440 nm to the rate of photosynthesis can be considered as additional evidence for a major contribution to the blue fluorescence by NADPH. These results indicate that the steady state concentration of NADPH is closely related to the rate of photosynthesis, and one would expect that the rate of photosynthesis is the limiting factor in the level of the steady state concentration of NADPH.

CONCLUSIONS

The prime contributor to the blue fluorescent band with a maximum at 440 nm is the water-soluble compound, NADPH. Other minor contributors are possibly vitamin K_1 and beta-carotene. Compounds of minimal solubility such as polyphenolics and lignins may also contribute to the blue fluorescence. The band at 525 nm appears to be caused by the presence of riboflavin.

The strong relationship between the magnitude of the blue fluorescence band and the rate of photosynthesis provided additional evidence of the major contribution to the blue fluorescence band by NADPH. An important ramification of this relationship is that the measurement of changes in the blue fluorescence band may prove to be useful as a means of the remote estimation of the rate of photosynthesis.

REFERENCES

Chappelle, E. W., and Williams, D. L. (1987), Laser induced fluorescence (LIF) from plant foliage, IEEE Trans. Geosci. and Remote Sens. GE-25:726–736.

Chappelle, E. W., Wood, F. M., McMurtrey, J. E., and Newcomb, W. W. (1984a), Laser induced fluorescence of green plants. 1. A technique for the remote detection of plant stress and species differentiation, Appl. Opt. 23:134–138.

Chappelle, E. W., McMurtrey, J. E., Wood, F. M., and Newcomb, W. W. (1984b), Laser induced fluorescence of green plants. 2. LIF changes caused by nutrient deficiencies in corn, Appl. Opt. 23:139–144.

Chappelle, E. W., Wood, F. M., Newcomb, W. W., and McMurtrey, J. E. (1985) Laser induced fluorescence of green plants. 3. LIF spectral signatures of five major plant types, Appl. Opt. 24:74–80.

Duysens, L. N. M., and J. Amesz (1957), Fluorescence spectroscopy of reduced phosphopyridine nucleotide in intact cells in the near-ultraviolet and visible region, Biochim. Biophys. Acta 24:19–26.

Duysens, L. N. M., and G. Sweep (1957), Fluorescence spectroscopy of pyridine nucleotide in photosynthesizing cells, Biochim. Biophys. Acta 25:13–16.

Forrest, H. S., Balen, C. V., and Meyers, J. (1957), Occurrences of pteridines in a blue-green algae, Science 125:699.

Fry, S. C. (1979) Phenolics components of the primary cell wall and their possible role in the hormonal regulation of growth, Planta 146:343–351.

Fry, S. C. (1982) Phenolic components of the primary cell wall, Biochem J. 203:493–504.

Goulas, Y., Moya, I., and Schmuck, G. (1990), Time resolved spectroscopy of the blue fluorescence of spinach leaves, Photosyn. Res., forthcoming.

Kok, B. (1976), Photosynthesis: The path of energy, in Plant Biochemistry, Academic, New York, pp. 846–883.

Lundquist, K., Josefsson, B., and Nyquist, G. (1978), Analysis of lignin products by fluorescence spectroscopy, Holzforschung 32:27–32.

Okamura, M. Y., Feher, G., and Nelson, N. (1982), Reaction centers, in Photosynthesis, Vol. 1, Energy Conversion by Plants and Bacteria, Academic, New York, 197–264.

Olson, J. M., and Amesz, J. (1986), Action Spectra for fluorescence excitation of pyridine nucleotide in photosynthetic bacteria and algae, Biochim. Biophys. Acta 37:14–24.

Olson, J. M., Duysens, L. M. N., and Kronenberg, G. H. M. (1959), Spectrofluorometry of pyridine nucleotide reactions in Chromatium, Biochim. Biophys. Acta 36:125–131.